AD_____

Award Number: DAMD17-01-1-0280

TITLE: Identification of Widely Applicable Tumor-Associated Antigens for Breast Cancer Immunotherapy

PRINCIPAL INVESTIGATOR: Jining Bai, Ph.D.

CONTRACTING ORGANIZATION: The Johns Hopkins University
Baltimore, Maryland 21205

REPORT DATE: October 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2004	Annual (15 Sep 2003 - 14 Sep 2004)

4. TITLE AND SUBTITLE
Identification of Widely Applicable Tumor-Associated Antigens for Breast Cancer Immunotherapy

5. FUNDING NUMBERS
DAMD17-01-1-0280

6. AUTHOR(S)
Jining Bai, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Johns Hopkins University
Baltimore, Maryland 21205

E-Mail: jnbai@jhmi.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

This study is a feasibility study of a novel immunotherapeutic strategy for the treatment of breast cancer. The rationale is based upon recent findings that genes belonging to the pp32 family are differentially and alternatively expressed in most human breast cancers. In general, benign breast tissues express pp32, a tumor suppressor, whereas breast cancers express tumorigenic family members, including pp32r1 and pp32r2. Since pp32r1 and pp32r2 are expressed in nearly all breast cancers, but not in normal adult tissues, they may reasonably serve as targets for antigen-specific immunotherapy. The purpose of this study is to identify tumor-associated antigens (TAA) in pp32r1 and pp32r2, then test their suitability *in vitro* as immunotherapeutic targets in breast cancer. Currently, the animal study is underway. If successful, the results may translate into eventual clinical trials of peptide vaccines or adoptive T cell therapy

14. SUBJECT TERMS
TAA, Immunotherapy

15. NUMBER OF PAGES
10

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

In the IDEA proposal, we proposed a feasibility study of a novel immunotherapeutic strategy for the treatment of breast cancer. The rationale is based upon recent findings that genes belonging to the pp32 family (Figure 1) are differentially and alternatively expressed in most human breast cancers. In general, benign breast tissues express pp32, a tumor suppressor, whereas breast cancers express tumorigenic family members, including pp32r1 and pp32r2. Since pp32r1 and pp32r2 are expressed in nearly all breast cancers, but not in normal adult tissues, they may reasonably serve as targets for antigen-specific immunotherapy.

Body:

Statement of Works:

Task 1. Identify, synthesize and test candidate peptides that could potentially bind to HLA class I molecules based on the coding sequence of pp32r1 and pp32r2. (Month 1-6)

Task 2. Screen *in vitro* for candidate pp32r1 & pp32r2 peptides that fulfill the requirements for TAA. (Month 7-12)

Task 3. Evaluate the pp32r1/pp32r2- specific cytotoxicity against a broad range of natural targets (established or primary breast cancer cell lines) to determine range of applicability.(Month 13-20)

Task 4. Evaluate *in vivo* immunogenicity of pp32r1 and/or pp32r2-derived TAAs in human breast cancer animal models. (Month 21-36)

In the first year of this project, we successfully identified two candidate TAA epitopes, which are capable of triggering MHC Class I dependent CTL response in vitro against artificial target cells. In the second year of this project, we further evaluated the applicability of the above candidate TAA epitopes against nature target cells (Task #3). However, due to the high homology among pp32 family members (over 90% identity at amino acid level), there is no existing methods to identify the subtype specific expression of pp32r1 and pp32r2. Therefore, it is challenging to identify the "true" nature target cells that express pp32r1 or pp32r2 at protein level. To address this challenge, in the third year of this study, we make effort to raise monoclonal antibodies against pp32r1 and pp32r2 that are subtype-specific. We hope this alternative approach will allow us to screen "true" nature target cells to successfully carry-out Task#3. In addition, taking the lesson from Task#3, we also established an modified animal models for *in vivo* study (Task #4)

1) Task #1: Identify, synthesize and test candidate peptides that could potentially bind to HLA class I molecules based on the coding sequence of pp32r1 and pp32r2.

Using Bioinformatics and ImmunoGenetics tools, we analyzed the entire coding region of pp32, pp32r1 and pp32r2 genes for binding affinity with HLA-A*0201 molecule as well as the degradation pattern by proteasomal cleavages. The result of calculation shown (Table 1) that 19 motifs are potentially favorable of binding to HLA-A*0201 molecule with high affinity. To verify the prediction *in vitro*, HLA-A*0201+ TAP-deficient T2 hybridoma (ATCC) was pulsed with 50ug/ml of each peptide representing the motif (or control) and 5ug/ml of b2-microglobulin for 18hr at 37 C. HLA-A*0201 expression was then measured by flow cytometry using mAb BB7.2 (ATCC) followed by incubation with FITC-conjugated secondary antibody. Fluorescent index of HLA-A*0201 to each peptide can be determined as: (mean fluorescence with peptide - mean fluorescence without peptide) / (mean fluorescence without peptide). The result shown 10

out of 20 motifs is capable of binding to HLA-A*0201 in a concentration dependent manner (Table 1).

2) Task #2: Screen for candidate pp32r1 & pp32r2 peptides that fulfill the requirements for TAA. In order to be qualified as a TAA, a motif has to be able to meet several criteria in addition to the binding to HLA-A*0201. These requirements include (i) the antigen can be naturally processed by tumor cells, (ii) it permits expansion of antigen-specific CTL; (iii) it is presented in a MHC-restricted fashion. CTL assay was carried out to test if the motifs identified in Aim#1 fulfill the requirements for TAA.

In brief, Cr^{51}-labeled target cells (T2 cells pulsed with peptide or cancer cell expressing pp32 family members) were incubated with various numbers of CTL effecter cells for 4 hr. Cr^{51}-release assays were performed in triplicate per condition using $5x10^3$ labeled target cells per well in a 96-well plate. Percent specific lysis will be calculated from CPM of (experimental result - spontaneous release)/(maximum release - spontaneous release). The results, summarized in Table 2, indicate that 2 out of 10 motifs fulfilled the above requirement as TAA.

3) Task #3. Evaluate the applicability of the pp32r1/pp32r2- specific cytotoxicity against a broad range of natural targets.

To evaluate the applicability of the pp32r1/pp32r2- specific cytotoxicity against a broad range of natural targets, primary cultures of breast tumor that are both HLA-A*0201 positive and pp32r1 / pp32r2 positive was selected as target cells. The expression of HLA-A*0201 was verified by flow cytometry, whereas the expression of pp32r1 and/or pp32r2 was confirmed by subtype-specific RT-PCR. CTL assay was carried out to test if the motifs identified in Task #1&#2 are applicable to HLA-A*0201 positive and pp32r1 / pp32r2 positive primary cultures. In brief, Cr^{51}-labeled target cells were incubated with various numbers of CTL effecter cells for 4 hr. Cr^{51}-release assays were performed in triplicate per condition using $5x10^3$ labeled target cells per well in a 96-well plate. Percent specific lysis will be calculated from CPM of (experimental result - spontaneous release)/(maximum release - spontaneous release). Unlike the artificial target cells used in Aim#2, the results shown no detectible pp32r1/pp32r2- specific cytotoxicity against primary cultures of breast tumor that are both HLA-A*0201 positive and pp32r1 / pp32r2 positive. A possible explanation might be the difference in expression/presentation of pp32r1 / pp32r2 between primary cells and artificial target cells.

Due to the high homology among pp32 family members (over 90% identity at amino acid level), none of the existing antibodies is subtype- specific. Therefore, the reliable method to screen pp32r1 and pp32r2 expression has been based on RT-PCR. Although this screen method is very effective to identify cells/tissue that express pp32r1 and pp32r2 at mRNA level, its result may not correlate with the expression of pp32r1 and pp32r2 at the protein level, which is crucial for evaluating pp32r1/pp32r2- specific cytotoxicity.

As an alternative, current efforts in the third year of the study are being made to establish subtype- specific antibodies so that a reliable method to test the expression of pp32r1 / pp32r2 at protein level will be available to re-evaluate the applicability of the pp32r1/pp32r2- specific cytotoxicity against natural target cells. In brief, cDNA of pp32r1 and pp32r2 are cloned into pEGX2 vector. The resulted GST-fusion constructs are expressed in BL21 E.Coli. GST-pp32r1 and GST-pp32r2 fusion proteins are purified via Glutathione affinity columns. The purified fusion proteins are used as antigens to immunizing animals. The subtype-specificity of the antibodies were determined by western blot against GST, pp32, pp32r1 and pp32r2. While majority of the

clones that screened so far were not subtype specific, we were able to identify one clone that appears to specifically recongnizing pp32r2 on western blot. Currently, we are doing another round of screen intended to identify subtype specific-antibodies against pp32r1.

4) Specific Aim 4. Evaluate *in vivo* immunogenicity of pp32r1 and/or pp32r2-derived TAAs in human breast cancer animal models. This phase of study includes (i) evaluate whether the identified TAAs are capable of triggering the expansion of pp32r1/ pp32r2-specific CTL and antigen-specific CTL response *in vivo*, (ii) Study anti-tumor activity of pp32r1/pp32r2- specific CTLs in breast cancer xenograft model. Learning our lesson from Task#3, we modified the target tumor cells used in our animal models. We made GFP-pp32r1 and GFP-pp32r2 fusion constructs in mammalian expression vector pEGFPN1. Stable transfectants with GFP-pp32r1 and GFP-pp32r2 expression were isolated by FACS selection. By using these stable transfectants in our breast cancer xenograft model, we hope that (i) that the problem in Task#3 can be circumvented and the tumor cells and its metastasis are more easily being traced during in vivo study.

Key Research Accomplishments:

We have identified two peptide motifs from pp32 family members, which fulfill the requirement to be TAAs. This study provided bases for further feasibility study of pp32r1 and pp32r2 as target breast cancer immunotherapy.

Reportable Outcomes:

The result of Specific Aim #1 and #2 were presented at 2002 Era of Hope Department of Defense Breast Cancer Research Program Meeting.
Yu, W., Jagun, A., Zhu, X., Jaffee, EM, & Bai, J. Identification of Candidate Tumor-Associated Antigens from pp32 Family Members. *Era of Hope* (BCRP): 3:54-2, 2002.

Conclusions:

We demonstrated *in vitro* that

(i) the oncogenic pp32 family members can be presented by HLA-A*0201,
(ii) the HLA-A*0201 cells bearing these motifs can be recognized and lyzed by pp32r1- or pp32r2- specific CTL in a MHC class I specific manner.

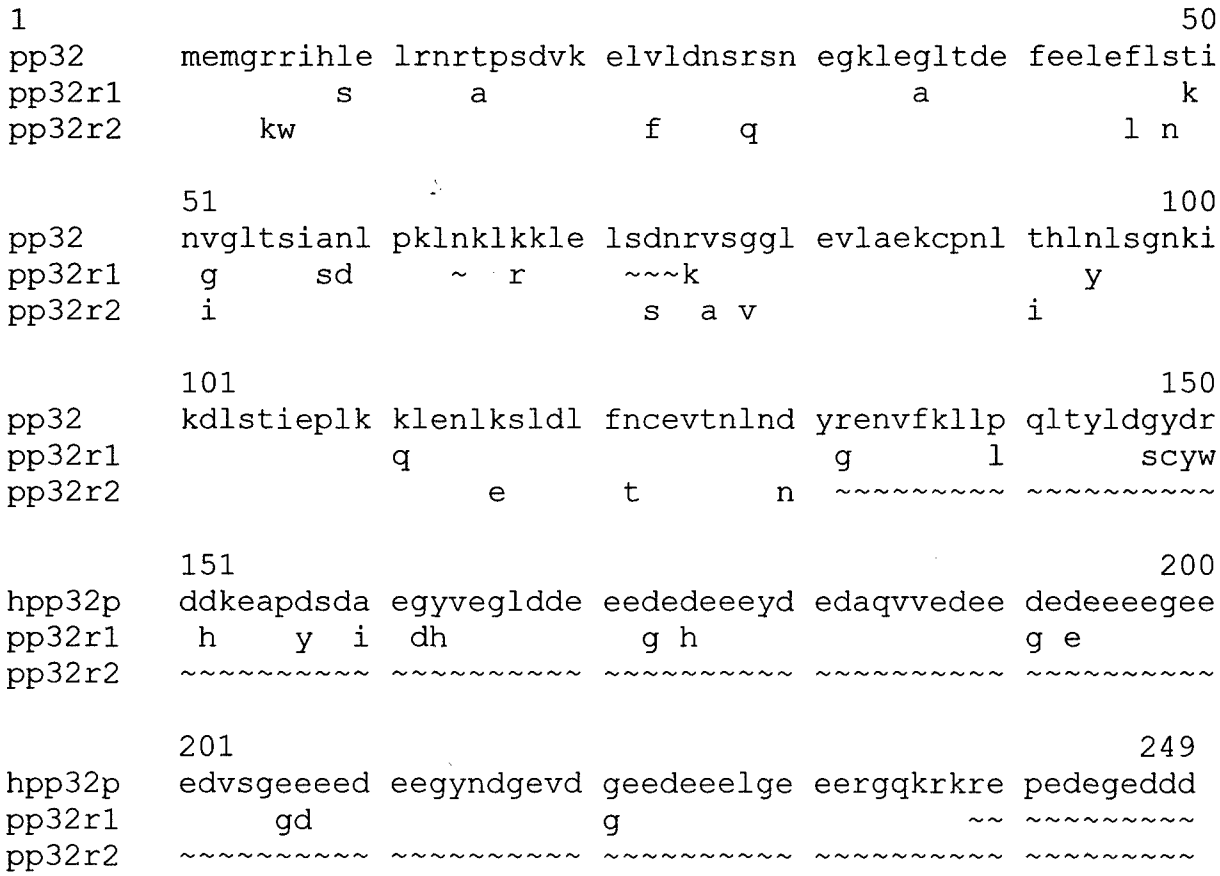

Figure 1. Alignment of pp32, pp32r1 & pp32r2 sequences.
Differences from the pp32 sequence are indicated underneath. The variant pp32r2 encodes a truncated protein (wavy lines indicate the truncated region).

Peptide	BIMAS	LpRep	FPEITHI	T2 Stabilization
0202-01	3499.535	3.37	26	+
0202-02	1591.602	2.46	22	-
0202-03	805.719	2.76	27	+
0202-04	681.542	3.54	18	+
0202-05	636.316	4.19	25	+
0202-06	481.542	6.90	27	-
0202-07	445.216	3.13	26	+
0202-08	432.319	4.87	21	-
0202-09	399.682	7.69	23	+
0202-10	379.216	5.81	13	-
0202-11	301.331	3.12	27	+
0202-12	281.542	3.47	22	-
0202-13	264.498	6.72	24	+
0202-14	226.014	3.54	20	-
0202-15	212.775	6.43	19	+
0202-16	172.752	6.81	21	+
0202-17	148.896	5.87	24	-
0202-18	139.730	6.72	19	-
0202-19	105.719	7.99	18	-
0202-20	103.362	6.79	21	-
MGA1	734.189	4.86	26	+

Table 1. Predicted HLA-A*0201 Binding Motifs and Their Ability to Bind T2 Cells.

Potential motifs was predicted by *BIMAS, LpRep, FPEITHI.*
The binding of Peptides to Human HLA-A2 was measured by T2 stabilization assay
Positive – calculated fluorescent index greater than 1.0.
Calculated fluorescent index = (Mean fluorescence with peptide - mean fluorescence without peptide)/(mean fluorescence without peptide)

Peptide	CTL Lysis*	Processing+	MHC I Restriction#
0202-01	+	n/a	n/a
0202-03	+++	Yes	Yes
0202-04	+	n/a	n/a
0202-05	+	n/a	n/a
0202-07	+++	Yes	Yes
0202-09	+	n/a	n/a
0202-11	-	n/a	n/a
0202-13	+	n/a	n/a
0202-15	-	n/a	n/a
0202-16	-	n/a	n/a
MGA1	+++	Yes	Yes
ID9	-	No	No

Table 2. Summary of CTL Assays for Motifs That are Capable of Binding to HLA-A*0201

Cytotoxicity Assay was carried out against Target cells:
* T2 Cell +/- peptides
+ MCF-7 ($A2^+$, $pp32r1^+$, $pp32r2^+$)

LNCAP ($A2^+$, $pp32r1^-$, $pp32r2^-$)
MCF-7 (+/- anti-HLA-A2mAb)

Summary of Personnel Partially Supported by This Idea Award:

1) Jining Bai (PI)
2) Tianzhi Mao (Technician)